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Difluoromethylornithine Antagonizes Taxol Cytotoxicity in MCF-7 Human Breast Cancer Cells

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Taxol is a naturally occurring anticancer agent. We studied the combined effects of taxol with 0.1 mM of the ornithine decarboxylase inhibitor α-difluoromethylornithine (DFMO) in the MCF-7 human breast adenocarcinoma cell line. The effects of taxol on MCF-7 cells were evident at 0.05-1 μM and the half-maximum inhibition was calculated to be 0.05 μM. Although the cells in the control group continued to proliferate during an 8-day growth period, cells in the taxol-treated group showed approximately 78% inhibition on day 6 and approximately 92% inhibition on day 8. The combined effects of different concentrations of taxol with 0.1 mM DFMO for 48 h showed that DFMO reversed the cytotoxicity of taxol. The combined effects of 0.5 μM taxol and 0.1 mM DFMO over an 8-day period resulted in the reversal of taxol cytotoxicity by 74% on the sixth day of culture. Pretreatment and posttreatment with 0.1 mM DFMO protected the MCF-7 human breast adenocarcinoma cells from the cytotoxic effect of taxol. Polyamine levels were inhibited in cells treated with DFMO for 24 h. In a separate experiment, we verified that the addition of exogenous putrescine along with taxol and DFMO to cultures for 48 h restored the cytotoxic effects of taxol. Following exposure to 0.5 μM taxol, over 59% of MCF-7 cells were in G_2/M phase. DFMO (0.1 mM) showed only a slight increase in the G_1 phase of the cell cycle. However, in cells treated with taxol and DFMO, there was no change in the percent of cells in the G_2/M phase compared to taxol-treated cells. Therefore, depletion of cellular polyamines may not interfere with cell cycle changes induced by taxol. Treatment of MCF-7 cells with 0.5 μM taxol resulted in the fragmentation of genomic DNA, indicating apoptosis, whereas the combined effects of taxol with DFMO inhibited DNA fragmentation.

Key words: Taxol; Difluoromethylornithine (DFMO); MCF-7 cells; Polyamines; Cell death; Cell cycle

Taxol, a diterpene initially isolated from the bark of the pacific yew *Taxus brevifolia*, is one of the most promising anticancer agents developed during the past decade (1). It suppresses the microtubule dynamics required for chromosome movement during mitosis, stalling cell division at metaphase-anaphase transitions. It has also been chemically synthesized (2). Taxol has been shown to have activity against breast and lung tumors (3) and has been approved for use in the treatment of refractory ovarian cancer (4) and metastatic breast cancer (5,6). Although the exact mechanism of taxol cytotoxicity is unknown, there is evidence that its antitumor effects result from binding to tubulin and preventing the disaggregation of microtubules (7).

DL-α-Difluoromethylornithine (DFMO[2]) is a highly specific inhibitor of polyamine biosynthesis and it results in the rapid depletion of putrescine and spermidine (8). The polyamines are essential for growth and differentiation and in some cases for cell survival. Application of DFMO to antineoplastic chemotherapy is partially focused on combination of the ornithine decarboxylase inhibitors with other antitumor agents (9).

To further expand on these observations, we investigated the effects of DFMO and taxol on the cultured human MCF-7 adenocarcinoma cell line. We started with a combination of taxol and DFMO to see if there might be a potentiation of cell kill in DFMO-treated cells. In contrast, the data presented in this report show that there is a marked resistance to cytotoxic effects of taxol when given in combination with DFMO.

MATERIALS AND METHODS

Chemicals

All of the tissue culture chemicals [RPMI-1640, antibiotics (streptomycin and pencillin), sodium bicarbonate, and taxol (pacilitaxel)] were purchased from Sigma Chemical Co. (St. Louis, MO). Dansyl-chloride, propidium iodide, RNase A, 4-6,diamidino-2-phenyl-inodole (DAPI), putrescine, spermine, and spermidine were also obtained from Sigma Chemical Co. DFMO was kindly provided by Marrion-Merrell Dow Research Center (Cincinnati, OH). [^{3}H]Thymidine was obtained from the Bhabha Atomic Research Centre (Bombay, India). Fetal calf serum (FCS) was obtained from Biological Industries (Kibbutz Beit Haemek, Israel). All other chemicals used were of analytical grade.

Cell Cultures

MCF-7, a human breast adenocarcinoma cell line, was obtained from the National Facility for Animal Tissue and Cell Culture (NFATCC) (Pune, India).

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[2]Abbreviations used: DFMO, DL-α-difluoromethylornithine; DAPI, 4-6,diamidino-2-phenyl-inodole; BCNU, carmustine.

The cell line was regularly maintained in RPMI-1640 medium supplemented with 0.2% sodium bicarbonate, 10% FCS, and antibiotics (50 μg/ml of pencillin and 100 μg/ml of streptomycin). The cells were routinely grown in 25-cm² flasks under an humidified atmosphere of 95% air and 5% CO_2 at 37°C and were subcultured until growth reached near confluency. For drug studies and for other experiments, cells were maintained in 60-mm petri dishes. A number of 60-mm petri dishes were plated with approximately 10^5 cells in medium for 48 h. The drugs were added 48 h after plating. The time of treatment of drugs varied with each experiment setup as indicated in the figure legends. Following exposure to drugs, the cells were rinsed and trypsinized. Cell growth was monitored by the counting of viable cells after trypan blue dye exclusion test using a hemocytometer. Each experiment was repeated at least three to four times and each treatment was in triplicate. The percentage of trypan blue excluded cells (live cell) was more than 95% in the untreated group.

Processing of Cell Pellets for Measurement of Polyamines

All procedures were performed at 4°C. Cells were harvested from plates by trypsinization. Cells were centrifuged and washed with ice-cold phosphate-buffered saline (PBS) to remove trypsin. The cells were then resuspended to give a final concentration of about 10^6 cell/ml. For polyamine assay, cells (approximately 10^6 cells) were resuspended in 250 μl of 2% perchloric acid and kept for 24 h at 4°C.

Polyamine Measurements

Cells were pelleted from perchloric acid. The supernatants were used for polyamine estimation. Dansyl derivatives were prepared according to Seiler (10) and polyamines were separated by thin-layer chromatography (TLC) on 0.2-mm-thick silica gel G plates, using ethylacetate/cyclohexane (2:3, v/v) as the solvent. Quantification of polyamines was accomplished using a Camag TLC Scanner with the TLC II software program Cats 3 (Camag, Sonnenmattstr, Switzerland). The concentration of unknown samples was determined against standard polyamines.

Cell Cycle Analysis

Cells were grown and treated with drug(s) for 48 h, harvested by trypsinization, washed with cold PBS, and pelleted by centrifugation at 2000 × *g* for 10 min at 4°C. The pelleted cells were fixed using ice-cold 70% ethanol for 24 h. Cells were centrifuged again at 2000 × *g* for 10 min at 4°C. Ethanol was removed without disturbing the pellet and 1.0 ml of propidium iodide staining solution (50 μg/ml of propidium iodide, 100 μg/ml of RNase A, and 1 mg/ml of glucose in PBS) was added to the cells with continuous vortexing. The cells were incubated for 30 min at room temperature. Samples were examined using EPIC® XL-software (Coulter Corporation, Miami, FL) and then analyzed using MULTICYCLE software (Phoenix Flow Systems Inc., San Diego, CA) for cell cycle analysis.

Cell Morphology

MCF-7 cells were exposed to 0.1 mM DFMO, 0.5 μM taxol, or DFMO and taxol for 24 h. Some of the cells were treated with 1.0 μM putrescine in addition to DFMO and taxol. At the end of the experiment, cells were washed with PBS, fixed in 70% ethanol, and stained with 10 μg/ml DAPI. Alterations in cell nuclei were photographed under a fluorescence microscope.

Quantitative Assay of DNA Fragmentation

Assays were conducted in triplicate in a flat bottom, 96-well microtiter plate in RPMI-1640 medium with 10% FCS in a total volume of 0.1 ml. Approximately 5 × 10^4 cells were seeded in each well. Wells for total counts received 0.1 ml of medium, whereas experimental wells received appropriate concentrations of drugs in medium. DNA fragmentations were measured by the release of [^{3}H]thymidine-labeled DNA fragments after a 24-h incubation. Cells were harvested after 24 h by adding 150 μl of 10 mM EDTA and 0.3% Triton X-100 to each well. Incorporated ^{3}H was measured by washing cells on glass microfiber filters and counting in a Beckman scintillation spectrometer (LS 1800 model, Beckman Instruments Inc., Palo Alto, CA). The percent of DNA

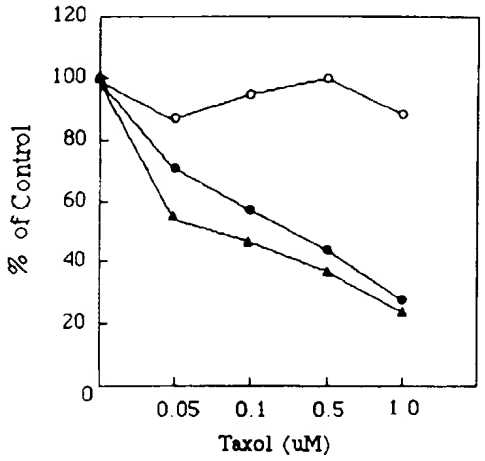

Figure 1. Effects of different concentrations of taxol individually and in combination with 0.1 mM DFMO on MCF-7 cells. Cells were plated in triplicate in 24-well growth plates (5 × 10^4 cells/well) in the medium. Two days after plating, the medium was replaced with the same medium but containing 0.1 mM DFMO or 0.05-1.0 μM taxol. To some of the cultures 1.0 mM putrescine was also added. Triplicate wells were counted for cell number after 48 h of drug treatment and the means were plotted. Taxol (●), taxol + DFMO (○), taxol + DFMO + putrescine (▲). Results are the means ± SD of three to four replicates.

released was calcualted as: % DNA fragmentation = (total cpm − experimental cpm)/total cpm.

All of the experiments were conducted in triplicate and repeated at least three to four times, and the average ± SD was calculated; SDs > 5% of the average value are not shown.

RESULTS

The cytocidal response of taxol on MCF-7 cells exhibited a concentration-dependent inhibition of growth. The half-maximum inhibition was calculated to be 0.05 μM (Fig. 1). DFMO also inhibited the proliferation of MCF-7 cells. The effects of DFMO were evident at different concentrations of DFMO (0.01–10 mM), as seen in Figure 2. The half-maximum inhibtion (IC_{50}) in the extent of proliferation has been seen at 0.05 mM DFMO.

The combined effects of taxol and DFMO on the growth of MCF-7 cells were studied using three different experimental sets. In the first set MCF-7 cells were treated with different concentrations of taxol along with 0.1 mM of DFMO. The combined effects of different concentrations of taxol with 0.1 mM DFMO resulted in a striking antagonism of the cytotoxic effects of taxol. Different concentrations of taxol, when used with 0.1 mM of DFMO to treat MCF-7 cells, showed a 100% increase in survival when compared to cells treated with taxol alone (Fig. 1). The survival curves for cultures treated with 0.1 mM DFMO along with 1.0 mM putrescine and different concentrations of taxol showed that exogenous putrescine reversed the DFMO-induced protection from the cytotoxicity of taxol and restored the survival curves to near those of controls (Fig. 1). The

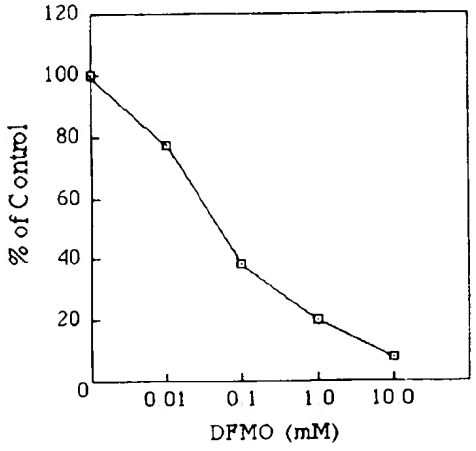

Figure 2. Effects of different concentration of DFMO on the rate of proliferation of MCF-7 cells. Cells were plated in triplicate in 24-well microtiter plates containing 5×10^4 cells/well. Two days after plating, the medium was replaced with the same medium but containing indicated concentration of DFMO. Triplicate wells were counted for cell number after 48 h and means were plotted.

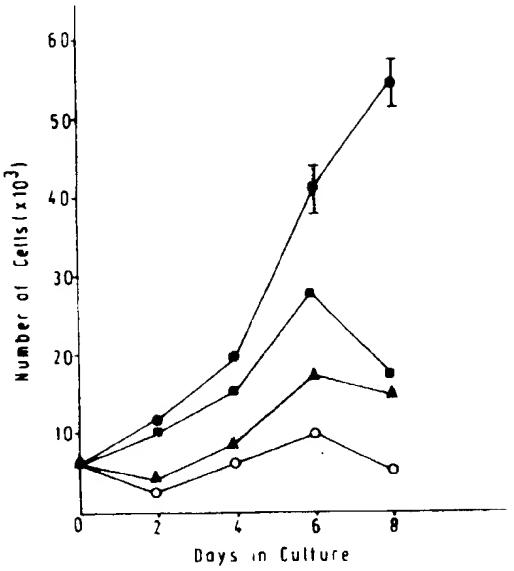

Figure 3. Effect of taxol and DFMO on the rate of proliferation of MCF-7 cells. Cells were plated in triplicate in 96-well microtiter plates. Two days after plating the medium was replaced with the same medium but containing 0.5 μM taxol, 0.1 mM DFMO, or both agents. Medium was changed every 2 days. Contol (●), 0.5 μM taxol (○), 0.1 mM DFMO (▲), taxol + DFMO (■). Triplicate wells were counted for cell numbers every 2 days and means were plotted.

combined effects of 0.5 μM taxol and 0.1 mM DFMO over an 8-day period of exposure are shown in Figure 3. Both taxol and DFMO independently inhibited the proliferation of MCF-7 cells. When combined, taxol and DFMO caused a reverse of the cytotoxicity on all of the days, with an increase in cell survival of 70% on day 6. However, on day 8 the extent of reversal was reduced by about 40%.

The second set involved pretreatment of cultures with 0.1 mM DFMO after 48 h of seeding 5×10^4 cell per well. After a final 24 h at 37°C, cultures were treated with taxol at indicated concentrations, harvested after 24 h, and counted. Pretreatment with 0.1 mM DFMO for 24 h significantly decreased taxol-induced cell kill at nearly every dose of taxol tested. However, pretreatment with DFMO induced a much lesser degree of protection from taxol in these cells when compared to the first set. Addition of 1.0 mM putrescine for 24 h to cultures that were pretreated with 0.1 mM DFMO for 24 h followed by treatment with indicated concentrations of taxol for 24 h after putrescine addition showed that exogenous putrescine reversed the DFMO-induced protection from taxol cytotoxicity and restored the survival curves to near those of controls (Fig. 4).

The third set involved addition of DFMO after taxol treatment (Fig. 5). In this set cells were treated with different concentrations of taxol for 24 h fol-

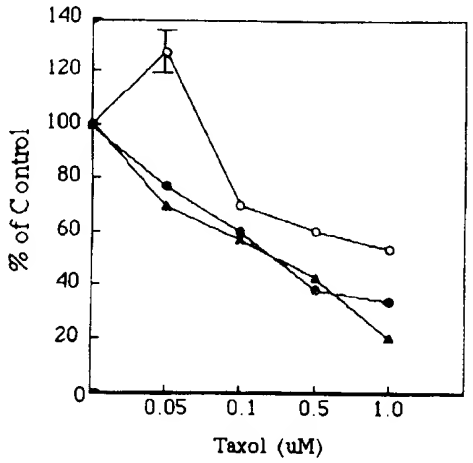

Figure 4. DFMO-induced protection of human breast adenocarcinoma cell line MCF-7 from taxol-induced cell kill and its reversal by exogenous putrescine. Cultures were pretreated with 0.1 mM DFMO at 48 h after seeding 5×10^4 cells. After an additional 24 h at 37°C, 0.1 mM putrescine was added to some of the DFMO-treated cultures. After a final 24 h at 37°C, cultures were treated with taxol at the indicated concentrations, harvested, and counted by trypan blue exclusion using a hemocytometer. Taxol (●), DFMO + taxol (○), DFMO + taxol + putrescine (▲). Bars represent the mean ± SD of three to four replicates.

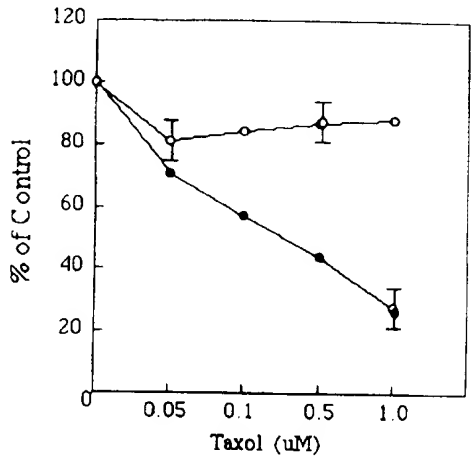

Figure 5. Effect of posttreatment of DFMO on taxol cytotoxicity in MCF-7 human breast adenocarcinoma cell line. Cells were plated in triplicate in 24-well plates (5×10^4 cells/well). Two days after plating cultures were treated with 0.5 μM taxol. After an additional 24 h at 37°C, 0.1 mM DFMO was added to some of the taxol-treated cultures. After a final 24 h at 37°C, cultures were harvested and counted by trypan blue exclusion method. Taxol-treated cells (●), taxol-treated cells subsequently treated with DFMO (○). Triplicate wells were counted for cell numbers and means were plotted.

lowed by addition of 0.1 mM DFMO for another 24 h. Cells were harvested in the taxol-alone group at 48 h after treatment with different concentrations of taxol. Addition of DFMO after taxol treatment resulted in reversal of cytotoxicity of taxol.

Treatment of MCF-7 cells with 0.1 mM DFMO for 24 h reduced intracellular concentration of putrescine and spermidine by about 97% and 70%, respectively, whereas the concentration of spermine increased compared to untreated cells (Table 1).

Cell cycle analyses were conducted to define the effects of 0.1 mM DFMO, 0.5 μM taxol, and DFMO + taxol on the cell cycle of MCF-7 cells. Cells were collected after 48-h exposure to these inhibitors. The results (Fig. 6, Table 2) show that the control cells had an S phase fraction of 13%. DFMO alone did not affect the percentage of cells in S phase, although a slight increase in G_1 phase cells was observed. Taxol blocked cells in G_2/M phase and lowered the percentage of cells in S phase. DFMO did not have any effect on the G_2/M arrest caused by taxol and it further decreased the percentage of cells in S phase fraction in taxol-treated cells.

The significant influence of taxol and DFMO on the growth of these cells led us to investigate whether the effect of these compounds was a result of triggering programmed cell death. The typical morphological changes of MCF-7 cell nuclei after 24-h incubation with 0.1 mM DFMO, 0.5 μM taxol, DFMO + taxol, or DFMO + taxol + 0.1 mM putrescine are shown in Figure 7. The DAPI staining shows apoptotic nuclei, either condensed or fragmented with both taxol (Fig. 7B) and DFMO (Fig. 7D). No apoptotic nuclei were observed when DFMO and taxol were given together (Fig. 7C). However, the addition of putrescine to MCF-cells along with DFMO and taxol resulted in the appearance of apoptotic nuclei (Fig. 7E).

Table 1. Polyamine Content of Cells Treated With DFMO

	nmol/10^6 Cells		
Treatment	Putrescine	Spermidine	Spermine
None	1.7 ± 0.06	5.7 ± 0.6	3.6 ± 0.1
DFMO (0.1 mM)	0.06 ± 0.006	1.7 ± 0.4	11.0 ± 1.1

Values are mean ± SD of three or four replicate determinations.

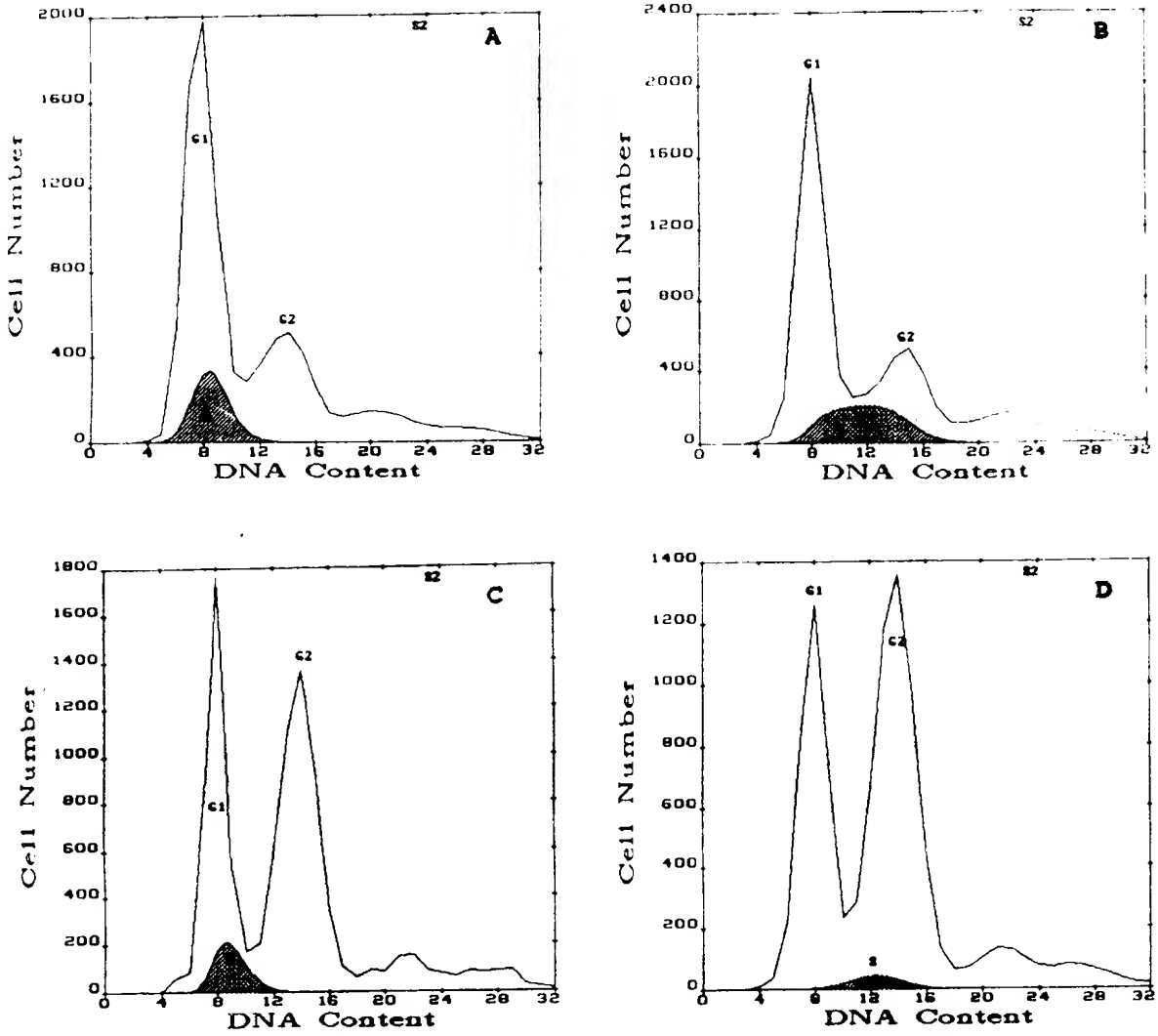

Figure 6. Flow cytometery analysis of MCF-7 cells after 48-h exposure to (A) control, (B) 0.1 mM DFMO, (C) 0.5 μM taxol, (D) 0.5 μM taxol + 0.1 mM DFMO.

Table 2. Flow Cytometric Analysis of MCF-7 Cells

	Distribution (%) of Cells in		
Treatment	G_1	S	G_2/M
None	55	13	32
Taxol (0.5 μM)	34	7	59
DFMO (0.1 mM)	60	20	21
Taxol (0.5 μM) + DFMO (0.1 mM)	39	2	59

Cell cycle phase distribution of human breast adenocarcinoma cell line MCF-7 after a 48-h treatment with taxol, DFMO, or DFMO and taxol. DFMO and taxol at the indicated concentrations were added to cultures 48 h after seeding approximately 10^5 cells/petri plate. Phase distributions were estimated by computer analysis of DNA histogram, obtained by flow cytometry of cells fixed with 70% ethanol and stained with propidium iodide.

To confirm the DNA fragmentation by these compounds [^{3}H]thymidine incorporation studies were done. Figure 8 demonstrates that 0.5 μM taxol and 0.1 mM DFMO, when given individually for 24 h, resulted in DNA fragmentation of MCF-7 cells. However, when given together no DNA fragmention was observed. Putrescine (1.0 mM) was found to cause DNA fragmentation of MCF-7 cells. When putrescine was given along with taxol and DFMO, DNA fragmentation was observed. The thymidine incorporation studies supported the morphological observations.

DISCUSSION

We have shown that DFMO given before, during, and after taxol treatment results in marked resistance to the cytotoxic effects of taxol. The addition of exogenous putrescine to the DFMO- and taxol-treated

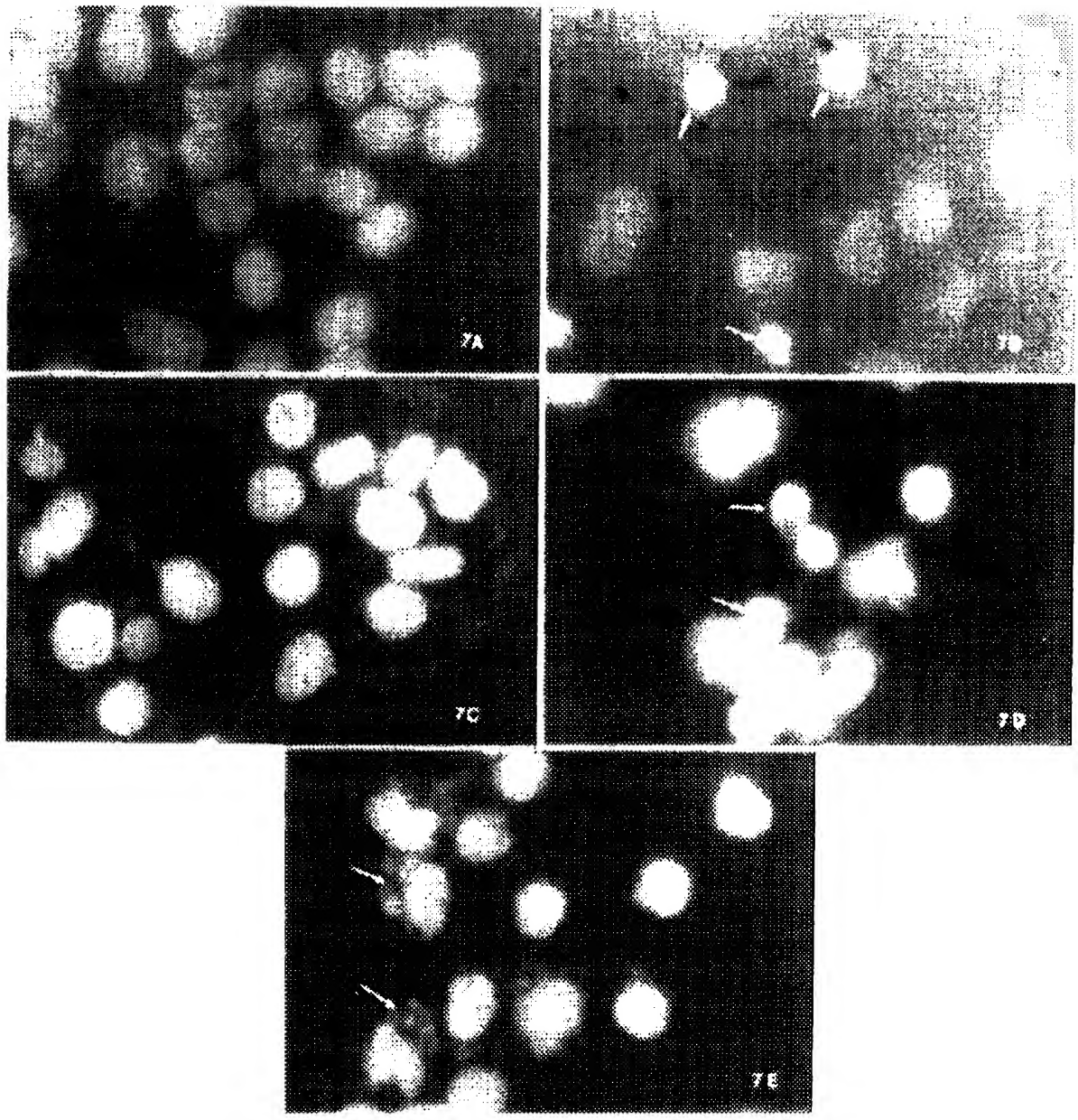

Figure 7. Morphological alterations of MCF-7 cell nuclei after incubation with taxol, DFMO, and putrescine. Cells were incubated in the presence of 0.5 μM taxol, 0.1 mM DFMO, DFMO + taxol or DFMO + taxol + 1.0 mM putrescine for 24 h. Cells were washed with PBS, fixed in ethanol, and stained with DAPI as described in Materials and Methods. The morphological changes in cell nuclei were observed under a fluorescence microscope. (A) Untreated control, (B) cells treated with taxol, (C) cells treated with DFMO and taxol, (D) cells treated with DFMO, (E) cells treated with DFMO, putrescine, and taxol. Arrows indicate MCF-7 cells with apoptotic nucleus (condensed or fragmented).

cultures restored the cytocidal response to taxol to near control levels.

These findings were unexpected, as we have used the concentration of taxol and DFMO in vitro to produce near additive effects on MCF-7 cell survival. Currently, the application of DFMO to antineoplastic chemotherapy is primarily focused on the combinations of the ornithine decarboxylase inhibitor with other antitumor agents. Earlier reports have shown that partial polyamine depletion induced by DFMO resulted in enhanced efficacy to carmustine (BCNU), a DNA alkylating and cross-linking agent (11). The molecular mechanism underlying this increased efficacy of BCNU is probably due to changes in the conformation of DNA in the polyamine-deficient cells, which may increase the accessibility of DNA to

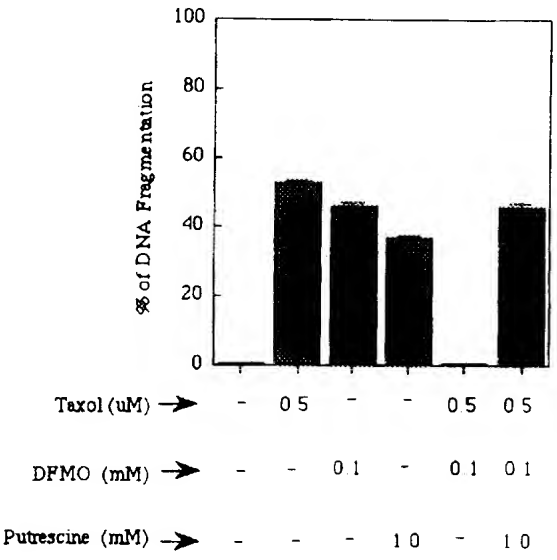

Figure 8. Fragmentation of DNA produced by taxol, DFMO, and putrescine. DNA fragmentation was measured as described in Materials and Methods after a 24-h incubation. Each point represents the mean ± SD of triplicate values.

BCNU. This enhanced DNA accessibility, though true for nitrosurea agents, does not extend to all other alkylating and cross-linking drugs (12).

An earlier report indicated that polyamine depletion decreases the cytotoxicity of another known anticancer agent, adriamycin (12). In the present study the pretreatment of MCF-7 cells with DFMO also antagonized the cytocidal effects of taxol, although to a much lesser degree when compared to the combined treatment of taxol and DFMO or post-treatment with DFMO. Our findings of putrescine reversal support the possibility of a corelationship between polyamine depletion and the reduced cytocidal efficacy of taxol.

The mechanism(s) responsible for the observed protection is unclear. A characteristic effect of taxol on cells has been to cause a block in the cell cycle at G_2/M. Over 59% of MCF-7 cells were blocked in G_2/M after exposure to 0.5 μM of taxol for 48 h. However, MCF-7 cultures treated with DFMO, which have partial response to cell cycle phase distributions when compared to controls, showed no alteration in the phase distribution of cells when DFMO was given along with taxol. Earlier reports on the altered cell cycle phase distributions in cultured human carcinoma cells partially depleted of polyamines by treatment with DFMO showed that MCF-7 cells showed a partial response to cell cycle phase distribution at the lowest concentration of 0.1 mM DFMO after 48-h treatment. Higher concentration of DFMO resulted in a marked increase in G_1 fraction and decrease in the S phase (13). Treatment with 0.1 mM DFMO in the present experiments decreased the fraction of cells in S phase after taxol treatment. This suggests that the mechanism of protection against taxol provided by polyamine depletion is not due to changes in the cell cycle. Earlier studies using adriamycin in ME-180 cultures showed that cultures treated with DFMO, which have cell cycle phase distribution no different from those of controls, show equivalent reductions in cytocidal efficacy of adriamycin as do other cell lines like Hutu-80 or A-427 (13). Here also it was reported that altered cell cycle phase distributions resulting from polyamine depletion may not be responsible for the observed protection to adriamycin.

The present study also demonstrated that both taxol and DFMO result in DNA fragmentation by 48% and 40%, respectively, as identified by thymidine incorporation studies. These studies were further confirmed by observing the morphological changes in MCF-7 cell nuclei after incubation with DFMO and taxol. However, when given together no fragmentation of DNA was observed. It is possible that DFMO inhibits apoptosis caused by taxol. Putrescine, when given along with DFMO and taxol, resulted in increased cell death that was comparable to taxol- or DFMO-alone-treated groups.

It is not clear what the exact mechanism of DFMO antagonism of taxol cytotoxicity might be. Treatment with DFMO caused depletion of polyamine levels. Earlier work by others demonstrated striking effects on microtubule disaggregation when cellular polyamine levels were depleted in CHO cells (14). It is possible that polyamine depletion in MCF-7 cells may affect the structure of the microtubules by involving the taxol binding site.

Thus, we conclude that treatment of MCF-7 human breast cancer cells with DFMO to deplete cellular polyamine levels protects the cells from the cytotoxicity caused by taxol. Exogenous putrescine resulted in a reversal of the protection by restoring the intracellular polyamine content. Depletion of cellular polyamines may not interfere with cell cycle changes induced by taxol. The resistance to taxol mediated by polyamine depletion could be due to prevention of microtubule condensation that occurs after exposure to taxol. We are pursuing additional studies to elucidate the mechanism of interaction between polyamine depletion and taxol.

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